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A Dual-Tropic Primary HIV-1 Isolate That Uses Fusin and the β-Chemokine Receptors CKR-5, CKR-3, and CKR-2b as Fusion Cofactors

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Summary

Here, we show that the β-chemokine receptor CKR-5 serves as a cofactor for M-tropic HIV viruses. Expression of CKR-5 with CD4 enables nonpermissive cells to form syncytia with cells expressing M-tropic, but not T-tropic, HIV-1 env proteins. Expression of CKR-5 and CD4 enables entry of a M-tropic, but not a T-tropic, virus strain. A dual-tropic primary HIV-1 isolate (89.6) utilizes both Fusin and CKR-5 as entry cofactors. Cells expressing the 89.6 env protein form syncytia with QT6 cells expressing CD4 and either Fusin or CKR-5. The β-chemokine receptors CKR-3 and CKR-2b support HIV-1 89.6 env-mediated syncytia formation but do not support fusion by any of the T-tropic or M-tropic strains tested. Our results suggest that the T-tropic viruses characteristic of disease progression may evolve from purely M-tropic viruses prevalent early in virus infection through changes in the env protein that enable the virus to use multiple entry cofactors.

Introduction

Human immunodeficiency virus type 1 (HIV-1) isolates exhibit marked differences in their abilities to infect CD4-positive cells. While all strains infect primary CD4+ T lymphocytes, most primary isolates from patients also infect primary macrophages (M-tropic), are typically nonsyncytium inducing (NSI), and fail to infect CD4+ transformed cell lines (Cheng-Mayer et al., 1988). Other isolates replicate well in CD4+ cell lines (T-tropic) and are syncytium-inducing (SI) but fail to infect macrophages. Changes in the biological properties of sequential viral isolates from infected individuals have been shown to correlate with disease progression. Thus, most viruses isolated from individuals shortly after seroconversion and during the asymptomatic phase of infection exhibit M-tropic NSI phenotypes (Conner and Ho, 1994; Conner et al., 1993; Roos et al., 1992; Schuitemaker et al., 1991, 1992; Tersmette et al., 1988, 1989a; Zho et al., 1993). With time, viruses emerge in many individuals that have T-tropic SI characteristics as well as increased replicative capacity. The appearance of T-tropic SI viruses correlates with progressive CD4+ lymphocyte decline and the development of AIDS (Tersmette et al., 1988, 1989a, 1989b). Dual-tropic viruses that retain the ability to replicate efficiently in macrophages in spite of having acquired SI characteristics have been described and may represent an important transitional phenotype leading to the T-tropic SI viruses associated with disease progression (Collman et al., 1992; Shibata et al., 1995).

In order to study the selective pressures that drive the transition from M- to T-tropism, the underlying source of permissiveness for M- and T-tropic viruses must be understood. HIV-1 cell tropism is determined largely at the level of virus entry and has been mapped to the envelope (env) protein, specifically to regions in the gp120 subunit that include the V3 loop (Cheng-Mayer et al., 1991; Hwang et al., 1991; Kim et al., 1995; Liu et al., 1990; O'Brien et al., 1990; Shioda et al., 1991; Westervelt et al., 1991, 1992). In order for HIV-1 to infect lymphocytes or macrophages, the env protein must bind to the primary viral receptor, CD4. While CD4 binding results in conformational changes in env (Sattentau and Moore, 1991), these are not sufficient to elicit the env-mediated membrane fusion reaction that is a prerequisite for virus infection. Rather, one or more cofactors are required in conjunction with CD4 for fusion to occur. The cofactor requirement is most clearly demonstrated by studies showing that expression of human CD4 (huCD4) in most nonhuman cells (and some human cell lines) fails to make them permissive for virus infection or env-mediated syncytia formation (Ashorn et al., 1990; Broder and Berger, 1995; Broder et al., 1993; Chesebro et al., 1990; Clapham et al., 1991; Dragic et al., 1992, 1995; Harrington and Geballe, 1993; Maddon et al., 1986). Nonpermissive CD4-positive cells can be made permissive for env-mediated membrane fusion and virus infection by transient heterokaryon formation with HeLa cells, indicating that one or more components (or cofactors) in HeLa cells can, when delivered to nonhuman cells, render the nonpermissive cells susceptible to HIV-1 infection (Broder et al., 1993; Dragic et al., 1992).

Recently, a seven-transmembrane domain protein, termed Fusin, has been shown to serve as a cofactor for T cell–tropic HIV-1 strains (Berson et al., 1996; Feng et al., 1996). Fusin exhibits approximately 30% homology with members of both the α and β-chemokine receptor families, though Fusin has not been shown to bind chemokines itself (Herzog et al., 1993; Jazin et al., 1993; Loetscher et al., 1994; Nomura et al., 1993). Expression of Fusin with huCD4 renders otherwise nonpermissive cell lines susceptible to env-mediated syncytia formation and virus infection (Berson et al., 1996; Feng et al., 1996). However, Fusin does not appear to be utilized as a cofactor by primary M-tropic NSI virus strains. Because of the similarity between Fusin and the chemokine receptors and the fact that the β-chemokines RANTES,

MIP-1α, and MIP-1β are the major HIV-1 suppressive factors secreted by CD8+ T cells (Cocchi et al., 1995; Paxton et al., 1996), we examined the ability of several chemokine receptors to function as cofactors for primary NSI viruses. We found that the β-chemokine receptor CKR-5, which shares 30% amino acid homology with Fusin and has been shown to bind RANTES, MIP-1α, and MIP-1β (Samson et al., 1996), could function as such a cofactor; expression of CKR-5 with huCD4 in otherwise nonpermissive quail and murine cells rendered these cells permissive for syncytia formation mediated by NSI, but not SI, virus env proteins as well as for infection by an M-tropic NSI virus. Furthermore, we found that a dual-tropic primary virus isolate, 89.6, could efficiently utilize both Fusin and CKR-5 as entry cofactors. In addition, the β-chemokine receptors CKR-3 and CKR-2b supported syncytia formation mediated by the 89.6 env protein, indicating that at least some primary virus isolates may utilize an impressive variety of related entry cofactors. The identification of CKR-5 as an entry cofactor for M-tropic HIV strains has important implications for understanding the molecular basis of viral tropism and HIV entry and suggests new possibilities for antiviral strategies. Our finding that HIV-1 89.6 can use both Fusin and CKR-5 as entry cofactors suggests that the evolution from a purely M-tropic NSI phenotype to a T-tropic SI phenotype may result from changes in HIV-1 env that first enable it to use both Fusin and CKR-5 as cofactors.

Results

The β-Chemokine Receptor CKR-5 Functions as a Cofactor for M-Tropic HIV-1 Strains

We have shown that expression of huCD4 in quail QT6 cells, like most other nonhuman cell lines, fails to render them permissive for HIV-1 infection and env-mediated syncytia formation. However, syncytia formation mediated by T cell–tropic (but not M-tropic) env proteins readily occurs when Fusin and huCD4 are coexpressed in QT6 cells (Berson et al., 1996). Expression of Fusin and huCD4 also makes otherwise nonpermissive feline, murine, human, mink, and simian cells suitable targets for T-tropic env-mediated syncytia formation (Berson et al., 1996; Feng et al., 1996). The QT6 cells were chosen for the current study because they can be efficiently transfected.

The similarity between Fusin and the chemokine receptors, coupled with the observations that the β-chemokines RANTES, MIP-1α, and MIP-1β inhibit replication of M-tropic HIV-1 strains in CD4+ T-cells (Cocchi et al., 1995; Paxton et al., 1996), prompted us to employ a syncytia assay to determine if the β-chemokine receptor CKR-5, which binds RANTES, MIP-1α, and MIP-1β (Samson et al., 1996), could function as a cofactor for M-tropic HIV-1 isolates. To do this, huCD4 and either Fusin or CKR-5 were transiently expressed in QT6 cells. After 24 hr, the target QT6 cells were mixed with HeLa cells that had been infected with recombinant vaccinia virus vectors that expressed the env proteins of the T-tropic strain BH8 or the M-tropic strains ADA or JR-FL. The cells were fixed, stained with methylene blue, and examined for syncytia formation 8 hr after mixing. As shown in Figure 1, HeLa cells expressing the T cell–tropic BH8 env protein readily formed syncytia with QT6 cells expressing huCD4 and Fusin but not with cells expressing huCD4 and CKR-5. In contrast, HeLa cells expressing M-tropic env proteins derived from JR-FL or ADA did not fuse with QT6 cells expressing huCD4 and Fusin (JR-FL) or fused very inefficiently (ADA). Only small isolated syncytia were observed with the ADA env protein (Figure 1). However, syncytia were readily observed with M-tropic env proteins when huCD4 and CKR-5 were coexpressed. Fusion was not observed in the absence of CD4 (data not shown) or when a noncleaved fusion-inactive form of env was used (Figure 1). Identical results were obtained when murine PA317 fibroblasts were used as targets (data not shown), indicating that the ability of CKR-5 to support M-tropic env-mediated syncytia formation was not cell type–dependent.

To examine syncytia formation more rigorously, we utilized a content-mixing assay (Broder and Berger, 1995; Nussbaum et al., 1994). In this assay, the target QT6 cells were transfected with plasmids encoding CD4 and either Fusin or CKR-5. In addition, the cells were infected with vCB21r, a recombinant vaccinia virus that expresses β-galactosidase under control of the T7 promoter. HeLa effector cells were infected with recombinant vaccinia viruses that expressed the desired env protein and with vTF1.1, a recombinant vaccinia virus that expresses the T7 polymerase. Target and effector cells were infected overnight and then mixed together and incubated at 37°C for 8 hr. If fusion occurs, the cytoplasmic contents of the target and effector cells mix, leading to β-galactosidase expression (Nussbaum et al., 1994). Fusion can therefore be monitored morphologically by scoring for in situ β-galactosidase activity and biochemically by lysing the cells and measuring β-galactosidase activity, using a quantitative colorimetric assay (Broder and Berger, 1995; Nussbaum et al., 1994).

As shown in Figure 2 and quantitated in Figure 3, HeLa cells expressing HIV-1 BH8 env protein formed syncytia with QT6 cells transfected with plasmids encoding huCD4 and Fusin, but not with cells expressing huCD4 and CKR-5. In contrast, HeLa cells expressing the M-tropic JR-FL env protein formed syncytia with QT6 cells expressing huCD4 and CKR-5 but not with cells expressing huCD4 and Fusin. Fusion was not observed when QT6 cells expressed huCD4 alone (data not shown) or with a noncleaved fusion-inactive form of env (see Figures 2 and 3). Thus, the β-chemokine receptor CKR-5 functions as a cofactor for M-tropic env-mediated membrane fusion.

CKR-5 Supports M-Tropic Virus Infection

To determine if expression of CKR-5 and huCD4 could render QT6 cells permissive for virus infection as well as fusion, CD4 and either Fusin or CKR-5 were transiently expressed in QT6 cells. After 24 hr, cells were infected with the T-tropic HIV-1 strain IIIB or the M-tropic strain JR-FL. The virus inoculum was removed and the cells harvested 24 hr later. To detect virus entry, we used a polymerase chain reaction (PCR)–based entry assay to

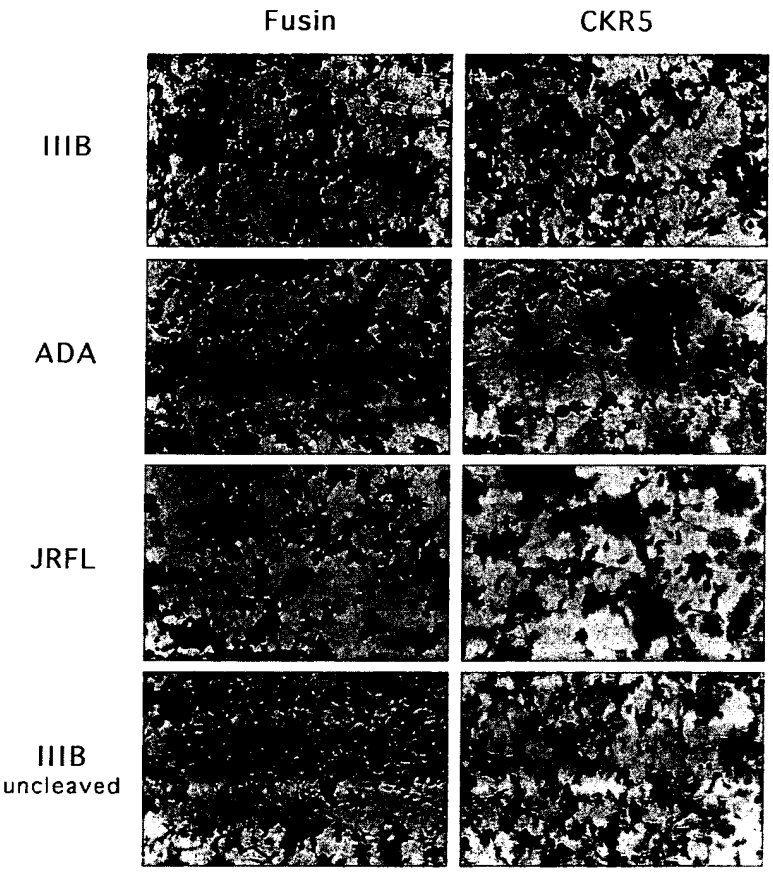

Figure 1. Syncytia Formation Detected by Methylene Blue Staining

QT6 target cells were infected with vCB3 (moi, 10) expressing CD4 and vCB21r (moi, 10) expressing the lacZ gene under the control of the T7 promoter. Within 2–4 hr postinfection, target cells were transfected with pSC59–Fusin expressing Fusin from the vaccinia synthetic early/late promoter, pCDNA3–CKR5 expressing CKR5 from the constitutive CMV promoter, or pCDNA3 vector. HeLa effector cells were infected with vaccinia (moi, 10) expressing the HIV-1 envelope proteins BH8, ADA, JR-FL, or a cleavage-defective form of the BH8 envelope. Effector cells were also infected with vaccinia vTF1.1 (moi, 10) expressing T7-polymerase under the control of the vaccinia late promoter. Cells were allowed to fuse for 8 hr before fixing with glutaraldehyde/formaldehyde and staining with methylene blue.

detect early viral DNA transcripts (Berson et al., 1996). As shown in Figure 4, HIV-1 IIIB entered QT6 cells that expressed both huCD4 and Fusin, consistent with our previous results (Berson et al., 1996). Entry was not observed when CKR-5 was expressed in place of Fusin. In contrast, HIV-1 JR-FL entered QT6 cells expressing huCD4 and CKR-5 but not cells expressing huCD4 and Fusin. Therefore, CKR-5 supported entry of an M-tropic

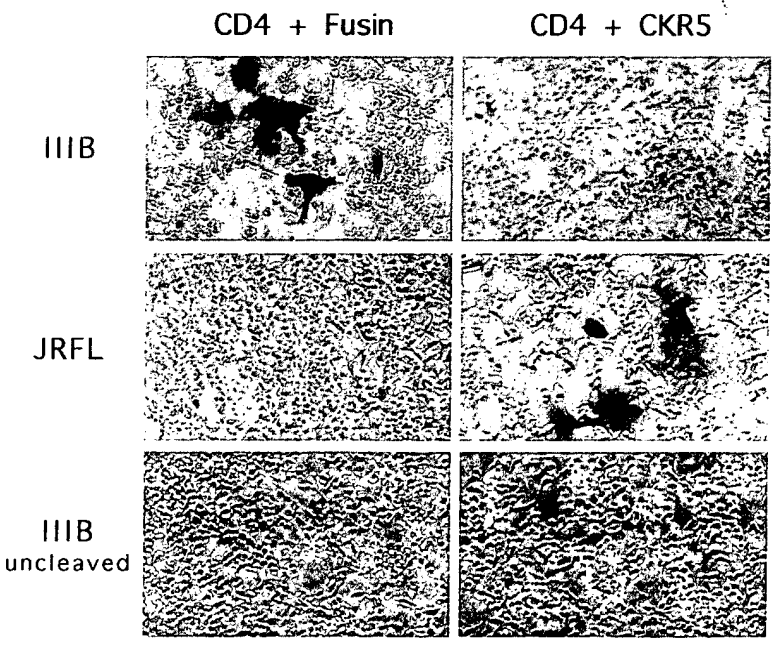

Figure 2. In Situ Detection of Syncytia Formation by Cytoplasmic Mixing and β-Galactosidase Expression

Fusion assays were performed using the same procedure as described in Figure 1, except that pREP8–Fusin expressing Fusin from the Rous sarcoma virus promoter was used in place of pSC59–Fusin. Cells were allowed to fuse for 8 hr before fixing in glutaraldehyde/paraformaldehyde and in situ staining with β-galactosidase substrate, as described in Experimental Procedures.

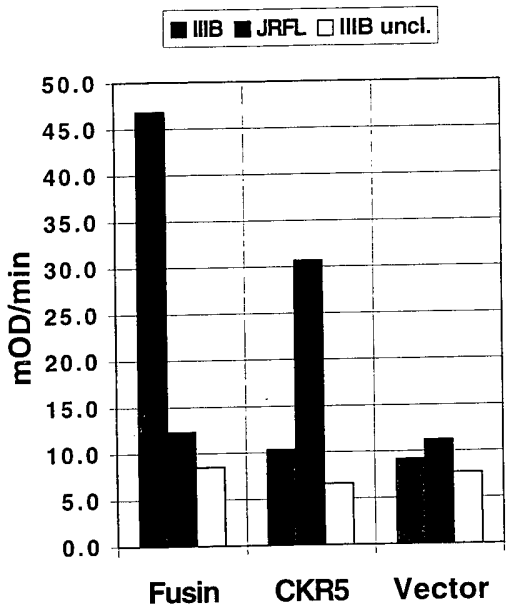

Figure 3. Quantitation of Fusion by β-Galactosidase Activity Assay

The fusion assay was performed using the same procedure as described in Figure 1. Cells were allowed to fuse for 8 hr before lysis in 0.5% NP-40. Cell lysates were harvested and assayed for β-galactosidase activity, as described in Experimental Procedures.

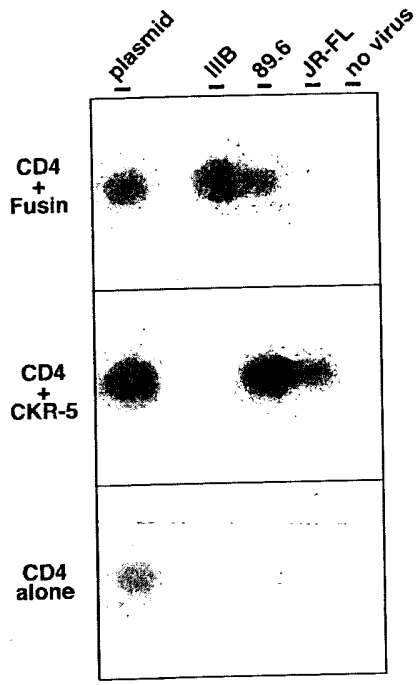

Figure 4. HIV-1 Infection of CD4-Expressing QT6 Target Cells

QT6 cells were cotransfected with pT4 and pSC59–Fusin, pCDNA3–CKR5, or pCDNA3 and then infected with HIV-1 strains BH8, 89.6, JR-FL, or mock supernatant. The following day, cells were lysed and HIV reverse-transcription products detected by PCR amplification of U3/U5 sequences, followed by Southern blot. HIV-1 plasmid served as a positive control. Quail-specific primers were used to demonstrate that equivalent amounts of DNA were used in each sample (data not shown). The small signal seen with 89.6 infection of QT6 cells expressing huCD4 alone was not reproducible and so most likely represents residual virus inoculum; other experiments showed no evidence for virus entry under these conditions.

but not a T-tropic virus strain, consistent with its ability to support membrane fusion mediated by M-tropic env proteins.

A Dual-Tropic HIV-1 Strain Utilizes Both Fusin and CKR-5

Most viruses isolated from asymptomatic individuals replicate in both macrophages and lymphocytes but do not induce syncytia formation in peripheral blood lymphocytes or in transformed cell lines (Conner and Ho, 1994; Conner et al., 1993; Schuitemaker et al., 1991, 1992). With disease progression, more cytopathic T-tropic SI viruses emerge. Whether cytopathic variants evolve from M-tropic NSI viruses or emerge from strains that are suppressed but present during the early stages of infection is not known. However, viruses with intermediate phenotypes have been described, supporting the idea that cytopathic viruses may evolve from noncytopathic strains via a transitional dual-tropic phenotype. We examined one such dual-tropic virus, 89.6, a primary virus isolate that retains the ability to replicate efficiently in macrophages in spite of having SI characteristics and the ability to replicate in some transformed T cell lines (Collman et al., 1992). To determine if the properties of 89.6 could be explained by the ability of 89.6 env to utilize multiple-entry cofactors, we tested the ability of the 89.6 env protein to fuse with QT6 cells expressing huCD4 and either Fusin or CKR-5. We found that HeLa cells expressing the env protein derived from 89.6 failed to form syncytia with QT6 cells that expressed huCD4 alone, indicating that this dual-tropic virus required one or more entry cofactors. In contrast, HeLa cells expressing 89.6 env readily formed syncytia with QT6 cells expressing huCD4 and either Fusin or CKR-5 (see Figure 5). Syncytia formation did not occur in the absence of CD4.

To obtain quantitative fusion data, we again used a gene reporter assay. Because the recombinant vaccinia virus that expresses the 89.6 env protein also constitutively expresses β-galactosidase, target QT6 cells were transfected with a plasmid containing luciferase, rather than lacZ, under control of the T7 promoter. Using this sensitive assay, we found that HeLa cells expressing the 89.6 env protein readily fused to QT6 target cells expressing huCD4 and Fusin and also to QT6 cells expressing huCD4 and CKR-5 (see Figure 6A). By contrast, HeLa cells expressing the T-tropic BH8 env protein formed syncytia only with cells expressing Fusin, while those expressing the M-tropic JR-FL env protein only fused with cells expressing CKR-5. Finally, we performed the PCR–entry assay to determine if both Fusin and CKR-5 could support virus entry into QT6 cells in conjunction with huCD4. As shown in Figure 4, we found that HIV-1 89.6 virus could use both Fusin and CKR-5 as entry cofactors, consistent with the membrane fusion results shown in Figures 5 and 6A. Thus, the dual-tropic nature of HIV-1 89.6 can be explained by its ability to use two distinct entry cofactors. A small signal was obtained when QT6 cells expressing huCD4 alone were infected with 89.6 (Figure 4), but this result was not

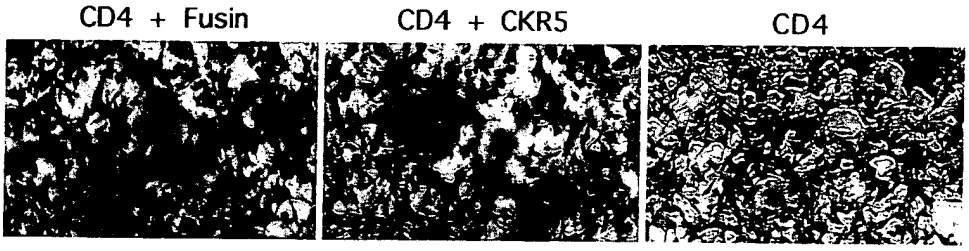

Figure 5. Syncytia Formation by 89.6 env Detected by Methylene Blue Staining

QT6 target cells were transfected with pREP8–Fusin expressing Fusin from the constitutive Rous sarcoma virus promoter, pCDNA3–CKR5 expressing CKR5 from the constitutive CMV promoter, or pCDNA3 vector. All target cells were also transfected with pT4, which constitutively expresses CD4 from the CMV promoter. HeLa effector cells were infected with vBD3 (moi, 10) expressing the 89.6 envelope. Cells were allowed to fuse for 8–10 hr before fixing with methanol and staining with methylene blue.

reproducible. Other experiments demonstrated no evidence of 89.6 entry into QT6 cells expressing huCD4 alone (consistent with the syncytia formation assay results in Figures 3 and Figure 6A), suggesting that this small signal was due to residual virus inoculum.

CKR-3 and CKR-2b Support 89.6 env-Mediated Membrane Fusion

The ability of HIV-1 89.6 to use both Fusin and CKR-5 as entry cofactors despite their divergent sequences (approximately 21% amino acid homology in the extracellular domains) prompted us to examine whether other chemokine receptors could function as cofactors for this dual-tropic virus. QT6 cells were transfected with plasmids encoding huCD4 as well as the β-chemokine receptors CKR-3 (which is expressed in eosinophils) and CKR-2b (which is expressed in monocytes). In addition, we transfected QT6 cells with a plasmid encoding the Duffy blood group antigen, a seven-transmembrane receptor that bears approximately 20% homology to Fusin and has been shown to bind to RANTES, IL-8, and MCP-1 (Chaudhuri et al., 1994; Neote et al., 1994; Peiper et al., 1995). Work by Dragic et al. (1995), demonstrating that fusion of human red blood cell ghosts with murine cells expressing huCD4 makes these cells susceptible for syncytia formation mediated by a T-tropic HIV-1 env protein, suggested that the Duffy antigen might serve as a HIV-1 cofactor. In the presence of huCD4, we found that both CKR-3 and CKR-2b supported syncytia formation by the 89.6 env protein (Figure 6B), but not by the BH8 and JR-FL env proteins. The Duffy blood group antigen failed to support membrane fusion by any of

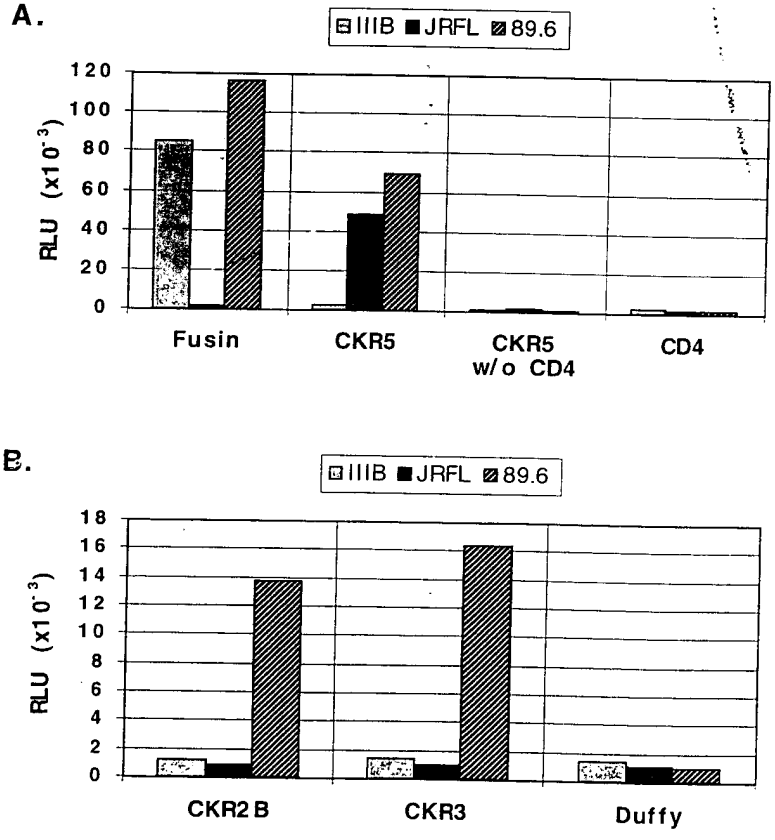

Figure 6. Quantitation of 89.6 Fusion by Luciferase Assay

(A) QT6 target cells were transfected with pREP8–Fusin expressing Fusin from the constitutive Rous sarcoma virus promoter, pCDNA3–CKR5 expressing CKR5 from the constitutive CMV promoter, or pCDNA3 vector. All target cells, except where indicated, were transfected with pT4, which constitutively expresses CD4 from the CMV promoter, and cells that did not receive CD4 were transfected with pCDNA3 vector. All target cells were also transfected with a plasmid containing the luciferase gene driven by a T7 promoter (Promega). HeLa effector cells were infected with vBD3 (moi, 10), which expresses the 89.6 envelope protein, or with viruses that express the JR-FL or BH8 env proteins. All effector cells were infected with vTF1.1 (moi, 10), which expresses T7 polymerase under the control of the vaccinia late promoter. Cells were allowed to fuse for 6 hr before lysis in Reporter Lysis Buffer (Promega) and assay for luciferase activity. Results are expressed in terms of relative light units (RLU), and raw data has been multiplied by 10^{-3}.

(B) This experiment was performed in an identical fashion to that shown in (A), except that the target cells were transfected with plasmids expressing CKR-3, CKR-2b, or the Duffy blood group antigen.

the env proteins tested. While the efficiency of fusion mediated by the different cofactors appeared to vary, the relative efficiency of Fusin, CKR-5, CKR-3, and CKR-2b to support syncytia formation must await development of specific antibodies that will allow quantitation of surface expression levels of these membrane fusion cofactors.

Discussion

HIV-1 isolates can be placed into three groups based on their ability to infect distinct target cell populations. Viruses that are M-tropic and that fail to form syncytia in culture represent the most prevalent phenotype isolated from individuals shortly after seroconversion and during the asymptomatic period of the disease (Conner and Ho, 1994; Conner et al., 1993; Roos et al., 1992; Schuitemaker et al., 1991, 1992; Tersmette et al., 1988, 1989b; Zho et al., 1993). By contrast, viruses that exhibit a T-tropic SI phenotype typically emerge later in infection and are associated with CD4+ T cell decline and progression to AIDS (Tersmette et al., 1988, 1989a, 1989b). Dual-tropic viruses retain the ability to replicate efficiently in macrophages despite exhibiting SI characteristics and may represent an important transitional phenotype in the evolution of T-tropic SI viruses from the M-tropic NSI viruses present early in the course of the disease (Collman et al., 1992; Shibata et al., 1995). An important factor governing cell tropism is the ability of a virus to enter different target cells. In the case of HIV-1, T-tropic viruses generally fail to enter macrophages, while M-tropic viruses often fail to enter transformed T cell lines in spite of the fact that both target cells express the primary viral receptor, CD4. Thus, CD4 by itself is not sufficient for virus entry. In addition, expression of huCD4 in nonhuman (and some human) cell lines typically fails to render them permissive for virus infection and env-mediated syncytia formation, supporting the idea that a cofactor in addition to CD4 is required for HIV-1 entry (Ashorn et al., 1990; Broder and Berger, 1995; Broder et al., 1993; Chesebro et al., 1990; Clapham et al., 1991; Dragic et al., 1992, 1995; Harrington and Geballe, 1993; Maddon et al., 1986). Only recently has a bona fide HIV-1 specific cofactor been identified, an orphan seven-transmembrane domain receptor termed Fusin (Berson et al., 1996; Feng et al., 1996).

Fusin is most closely related to the α- and β-chemokine receptor families (Federsppiel et al., 1993; Herzog et al., 1993; Jazin et al., 1993; Loetscher et al., 1994; Nomura et al., 1993). However, functional interactions between Fusin and various chemokines have not been detected (Herzog et al., 1993; Jazin et al., 1993; Loetscher et al., 1994; Nomura et al., 1993). Fusin exhibits a broad tissue distribution and is expressed at high levels in B-, T-, and monocyte-derived cell lines (Federsppiel et al., 1993; Herzog et al., 1993; Jazin et al., 1993; Loetscher et al., 1994; Nomura et al., 1993). Expression of Fusin and huCD4 in otherwise nonpermissive cell lines renders them susceptible to T-tropic env-mediated syncytia formation as well as infection by T-tropic SI viruses (Berson et al., 1996; Feng et al., 1996). However, Fusin either is not used by M-tropic NSI viruses as a cofactor or, as appears to be the case of the M-tropic strain ADA, is used very inefficiently (Berson et al., 1996; Feng et al., 1996). Given the high degree of homology between M- and T-tropic env proteins, we speculated that cofactors used by M-tropic viruses would be structurally related to Fusin. The similarity between Fusin and the chemokine receptor families, coupled with recent work showing that the β-chemokines RANTES, MIP-1α, and MIP-1β have significant antiviral activity against M-tropic but not T-tropic virus strains (Cocchi et al., 1995; Paxton et al., 1996), made the β-chemokine receptor CKR-5 a likely candidate.

We found that expression of CKR-5 in conjunction with huCD4 in otherwise nonpermissive quail QT6 or murine PA317 cells made these cells suitable targets for M-tropic env-mediated syncytia formation and also allowed virus entry. It is important to note that CKR-5 by itself did not support membrane fusion: fusion was only observed when target cells expressed both huCD4 and CKR-5. Expression of huCD4 and CKR-5 did not support fusion or virus entry by the T-tropic strain IIIB. CKR-5 contains 352 amino acids and has a predicted molecular mass of 40,600 kDa (Samson et al., 1996). It exhibits between 49% and 76% amino acid homology with other β-chemokine receptors, being most closely related to CKR-2b. Structural motifs include a single potential N-linked glycosylation site present in the third extracellular loop and four conserved cysteine residues in the ectodomain, with disulfide bonds predicted to occur between cysteines in the first and second extracellular loops and between the cysteine pair in the N-terminal domain and third extracellular loop (Samson et al., 1996; Strader et al., 1994). CKR-5 contains a serine/threonine-rich cytoplasmic domain that is a likely target for phosphorylation and contains a number of conserved proline residues in its transmembrane domains. Functionally, addition of RANTES, MIP-1α, and MIP-1β to cells expressing CKR-5 induces functional responses, though direct chemokine binding has not yet been demonstrated (Samson et al., 1996). CKR-5 shares approximately 30% amino acid homology with Fusin, with 38% homology between the transmembrane domains, 30% between the intracellular loops, and 21% between the extracellular domains. The first extracellular loop is the most highly conserved ectodomain region, with 7 of 14 residues being identical between Fusin and CKR-5.

Viruses representing evolutionary transitions between T- and M-tropic strains may be unique tools for linking disease manifestation with the underlying cellular basis of viral phenotype. The identification of Fusin as a cofactor for T-tropic viruses and CKR-5 as a cofactor for M-tropic viruses enabled us to determine if the dual-tropic nature of 89.6 could be explained by the use of both cofactors. HIV-1 89.6, isolated from peripheral blood mononuclear cells, replicates well in macrophages and in peripheral blood lymphocytes (Collman et al., 1992). However, unlike most M-tropic viruses, infection of peripheral blood lymphocytes with 89.6 results in syncytia formation. In addition, 89.6 can productively infect some transformed cell lines, such as CEMX174. We found that despite their divergent sequences, both Fusin and CKR-5 supported membrane

fusion mediated by the 89.6 env protein, and both supported 89.6 virus entry. The 89.6 env protein has 88%–91% amino acid homology with well-characterized M- and T-tropic viruses but does not exhibit greater homology to either group (Collman et al., 1992). The V3 loop of 89.6, a region of gp120 that has been shown to influence cell tropism, reflects this; it diverges almost equally from T- and M-tropic viruses. The ability of HIV-1 89.6 to utilize both CKR-5 and Fusin suggests that the cofactors share conserved conformational features or general structural characteristics, such as charge, that may be important for cofactor function. The ability of CKR-3 and CKR-2b to be used as fusion cofactors supports this idea and suggests that any env–cofactor interactions will be complex in nature. Alternatively, env proteins may harbor distinct sites that govern cofactor usage, and 89.6 may possess several such sites. Regardless, the ability of 89.6 to use both Fusin and CKR-5 suggests that relatively subtle changes in the env protein can result in the ability to use both entry cofactors. Such dual tropism could play an important role in the transition from M-tropic NSI to T-tropic SI HIV-1 strains during the course of disease. It will be interesting to determine if the high levels of RANTES, MIP-1α, and MIP-1β secreted by CD4+ lymphocytes from some HIV-1 infected individuals provide the selective pressure to drive the evolution of cofactor usage (Paxton et al., 1996).

The ability of 89.6 to utilize molecules as divergent as Fusin and CKR-5 as entry cofactors prompted us to determine if molecules more closely related to CKR-5 could likewise be used by this virus. The β-chemokine receptors CKR-3 (58% homology to CKR-5) and CKR-2b (76% homology to CKR-5) functioned as fusion cofactors for 89.6 env, while the Duffy blood group antigen (19% homology) did not. Expression of CKR-3 appears to be restricted to eosinophils (Combadiere et al., 1995b; Kitaura et al., 1996), making it unlikely that it could serve as an HIV-1 cofactor in vivo. However, more detailed studies on the distribution of CKR-3, as well as other chemokine receptors, will be needed. CKR-2b, however, is expressed in monocytes (Combadiere et al., 1995a) and so may be a biologically relevant cofactor for some HIV-1 strains.

The finding that fusion of either protease or heat-treated human red blood cell ghosts with murine cells expressing huCD4 rendered these cells competent for HIV-1 env-mediated membrane fusion indicated that red cell membranes contain one or more entry cofactors (Dragic et al., 1992; Puri et al., 1996). We do not yet know if human red cells express any of the known HIV cofactors, but the presence of the Duffy blood-group antigen, a promiscuous chemokine receptor, is intriguing. However, we found that coexpression of Duffy and huCD4 in quail cells failed to support syncytia formation by three different HIV-1 env proteins. While it is possible that red cells may contain an as yet unidentified cofactor, it is also possible that processing differences could account for these observations. The ability of Duffy to serve as a HIV-1 cofactor could also be tested by repeating the experiments of Dragic et al. (1992), using blood cells from individuals that lack the Duffy antigen.

Fusin and CKR-5 are likely to play a role in enabling the membrane-fusion reaction that is required for viral penetration, since expression of huCD4 alone in a non-permissive cell enables virus to bind but not to fuse with the target cell. A series of discrete steps must occur in order for HIV-1 to infect a cell. First, the virus must bind to the surface of the target cell, which generally involves a high affinity interaction between env and CD4. Next, a triggering event must elicit a conformational change in env that leads to membrane coalescence and fusion. Only after membrane fusion can the viral capsid enter the host cell cytoplasm. Therefore, the env protein plays two critical roles in viral entry: receptor binding and membrane fusion. While CD4 by itself is sufficient for binding env, it is not sufficient to trigger the entire series of conformational changes in env that are required for fusion to occur. Fusin and CKR-5 may provide the trigger that allows fusion to occur. A possible scenario is that conformational changes in env that result from CD4 binding could, in turn, allow env to interact with Fusin or CKR-5, leading to additional conformational changes that result in exposure of the fusion peptide and membrane fusion. It will be important to test this hypothesis and to determine if env interacts directly with the viral cofactors in either a CD4-dependent or -independent manner. The cofactors may also interact directly with CD4 and perhaps alter its conformation or presentation on the cell surface.

The identification of Fusin and CKR5 as entry cofactors has important implications for understanding viral entry, tropism, and pathogenesis, as well as the generation of transgenic animals that could serve as models for HIV infection. It also opens new areas for potential therapeutic strategies. The β-chemokines RANTES, MIP-1α, and MIP1-β have significant antiviral activity against M-tropic, but not T-tropic, HIV-1 strains (Cocchi et al., 1995; Paxton et al., 1996). The mechanism by which these chemokines exert their antiviral activity may be through receptor blockade or down-regulation. These or other compounds that prevent cofactor usage may be effective antiviral agents. It will also be important to determine if other proteins can serve as viral cofactors. Given the impressive genetic diversity exhibited by HIV-1 strains and the fact that 89.6 can use at least four distinct molecules as fusion cofactors, it will not be surprising if additional molecules may be able to function as cofactors for different HIV-1, HIV-2, and simian immunodeficiency virus strains. CKR-5, for example, is more closely related to other β-chemokine receptors than to Fusin, including CKR-1 (56% homology) which also binds RANTES, MIP-1α, and MIP-1β (Neote et al., 1993), and CKR-4 (49% homology) which binds to RANTES, MIP-1α, and MCP-1 (Power et al., 1995). The use of primary virus strains as well as viruses from different clades may reveal additional complexities. Finally, the ability of 89.6 to use both Fusin and CKR-5 as entry cofactors provides a molecular explanation for its dual-tropic phenotype and provides evidence that evolution of T-tropic SI viruses from M-tropic NSI viruses may involve a transitional phenotype that exhibits a broader range of cofactor usage.

Experimental Procedures

Constructs

pSC59–Fusin and pREP8–Fusin constructs have been previously described (Berson et al., 1996). CKR-5 was cloned into pCDNA3

(Invitrogen), using the upstream BamHI and downstream XBAI sites for expression using the cytomegalovirus (CMV) promoter. The luciferase–T7 plasmid was obtained from Promega. The plasmid pT4, providing expression of huCD4 under the control of the CMV promoter, was provided by Dr. Dennis Kolson (University of Pennsylvania). pTLZ, encoding lacZ under the control of the T7 promoter, was provided by Dr. Chris Broder (National Institute of Allergy and Infectious Diseases, National Institutes of Health). The 89.6 envelope gene was cloned into the vaccinia virus expression vector pSC59 by PCR. Internal regions of the gene were replaced with sequences not subject to PCR by restriction-fragment swaps, and all areas subject to PCR were confirmed by sequence analysis. The 5′ end of the clone was defined with PCR primer TCATTCGTCGACAGATTA ATTGATA, which introduces a SalI restriction site 46 bp before the 89.6 env start codon. The 3′ end of the clone utilizes the natural BspEI site approximately 600 bp after the 89.6 env natural stop codon. The SalI-to-BspEI sequences were inserted into pSC59 using the 5′ SalI and 3′ Xma sites in pSC59 and a BspEI–Xma linker sequence previously introduced into the vector.

Cells

The human cervical carcinoma cell line HeLa was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). The murine embryo fibroblast cell line PA317T4 (McKnight et al., 1994) was a gift from Dr. James Simon (University of Pennsylvania). The Japanese quail fibrosarcoma cell line QT6-C5 (QT6; ATCC# CRL-1708) was provided by Paul Bates (University of Pennsylvania). HuTK− 143B fibroblasts (#CRL-8303) were obtained from the American Type Culture Collection.

Tissue culture media and supplements were purchased from Life Technologies, Inc. unless otherwise noted. HeLa, PA317T4, and HuTK− were maintained in Dulbecco's modified Eagle's media: high glucose, supplemented with 10% fetal bovine serum (Hyclone). QT6 cells were maintained in Medium 199, supplemented with 10% tryptose phosphate broth (Sigma), 5% fetal bovine serum, and 1% chicken serum. All media were supplemented with 2 mM glutamine and penicillin–streptomycin.

Viruses

Dr. Christopher Broder of the NIAID kindly provided us with the following panel of recombinant vaccinia viruses encoding the envs of several HIV-1 strains (indicated in parentheses; Broder and Berger, 1995): vSC60 (IIIB, BH8 clone), vCB39 (ADA), and vCB28 (JR-FL). The recombinant vaccinia virus vCB16, encoding a nonfusogenic uncleaved BH8 env was used as a negative control. Dr. Broder also provided us with the recombinant viruses vCB3 (Broder and Berger, 1995), encoding huCD4; vTF1.1, encoding the T7 RNA polymerase; and vCB21r, encoding lacZ under control of the T7 promoter. The recombinant vaccinia virus vBD3, expressing the 89.6 full-length envelope protein under control of the vaccinia virus early/late promoter was generated using standard techniques utilizing thymidine kinase–negative selection (Earl, 1991). Vaccinia virus was created by transfecting the pSC59/89.6 gp160 plasmid into CV-1 cells infected with the wild-type vaccinia virus strain WR. Recombinants were screened by expression of lacZ and were plaque-purified five times to homogeneity.

Gene Reporter Fusion Assay

To quantitate cell–cell fusion events, we utilized a modified version of the gene reporter fusion assay described by Nussbaum et al. (1994). T7 RNA polymerase and env proteins were introduced into effector HeLa cells by infection with recombinant vaccinia viruses. Target QT6 cells were transfected with CD4, Fusin, or CKR5, as indicated in the text and legends. For quantitative assays, a reporter gene coding for either β-galactosidase or luciferase under the control of the T7 promoter was transfected into the target cells. Vaccinia-encoded proteins were produced by infecting cells at a multiplicity of infection (moi) of 10 for 1.5–4 hr at 37°C. Effector cells were then trypsinized, washed with phosphate-buffered saline, resuspended in media, and incubated at 32°C overnight in the presence of rifampicin. Proteins were generally introduced into target QT6 cells in 24 well plates by transfection of 2 μg of each plasmid, using the calcium-phosphate precipitation method. The transfectant was removed after 4 hr, and the cells were incubated at 37°C overnight. In some experiments, as indicated in the figure legends, CD4 and T7–β-galactosidase were introduced by infection with recombinant vaccinia viruses at a moi of 10. Cells were infected 2–3 hr prior to transfection. In infection/transfection experiments, overnight incubation was at 32°C in the presence of rifampicin. Transfection and infection/transfection of target cells yielded identical results, although infection experiments generally caused cytopathic effects and increased background.

To initiate fusion, target and effector cells were mixed in 24 well plates at 37°C in the presence of ara-C and rifampicin and allowed to fuse for 8–10 hr. Syncytia formation was monitored by fixing the cultures in 0.29% glutaraldehyde/1% formaldehyde/phosphate-buffered saline and staining with 0.5% methylene blue, 0.17% pararosaniline in methanol. To quantitate fusion, NP-40 was added to a final concentration of 0.5%, and aliquots of the cell lysates were monitored for β-galactosidase activity using the colorimetric assay described by Nussbaum et al. (1994). In some cases, β-galactosidase was detected by in situ staining. Since the 89.6 env-encoding vaccinia virus expressed the lacZ gene, quantitation of 89.6 env-mediated fusion was measured using a luciferase assay. After 8–10 hr of fusion, 2–3 × 10^5 cells were lysed in 150 μl of reporter lysis buffer (Promega) and assayed for luciferase activity according to the instructions of the manufacturer (Promega).

Infection Studies

pREP8–Fusin or pcDNA3–CKR5 were cotransfected with pT4 (2 μg of each plasmid) into 2 × 10^5 QT6 cells in 24 well tissue culture plates, using calcium-phosphate precipitation. The next day, cells were infected with DNase-treated (15 U/ml for 30 min at room temperature) cell-free virus stock for 4 hr at 37°C, using 75 ng p24 antigen each of strains JR-FL, 89.6, or IIIB (derived from clone HXB2). Following infection, cells were washed and fed. The cells were detached with trypsin 24 hr later, pelleted, suspended in 50 μl lysis buffer (100 mM KCl, 20 mM Tris [pH 8.4], 0.1% NP-40, 500 μg/ml proteinase K), and incubated for 2 hr at 60°C, followed by boiling for 15 min.

HIV detection by PCR was performed for 35 cycles on 2.5 μl of cell lysate to amplify a 430 bp region of U3/U5 LTR DNA sequences, using primers LTR-plus/LTR-minus (5′-ACAAGCTAGTACCAGTTGA GCC-3′; 5′-CACACACTACTTGAAGCACTCA-3′). Products were resolved by electrophoresis on 2% agarose gels, transferred to Hybond N+ (Amersham) and probed with a [^{32}P] end-labeled oligonucleotide probe (5′-ATCTACAAGGGACTTTCCCGC-3′) followed by autoradiography. To ensure that equivalent amounts of total cellular DNA were amplified, quail cell–specific primers Q101-plus/Q261-minus (5-GGTAACGGTTCTTTGTCCCGTTGC-3′; 5′-CGC GCTGGTCCCGCAGCCCCACTC-3′) were used to amplify a 153 bp region of the avian sarcoma virus receptor tvA, which is present in QT6 cell genomic DNA (Bates et al., 1993), which was visualized by ethidium bromide staining.

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